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Opsonins in normal mouse serum for the phagocytic killing of *Proteus mirabilis* by murine neutrophils

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Summary. An assay of phagocytic killing by murine neutrophils in homologous serum was used to determine the nature of the opsonins in normal mouse serum for phagocytic killing of *Proteus mirabilis*. Leucocytes from the peritoneal cavities of mice given an intraperitoneal inoculation of brain–heart infusion broth 3 h previously, phagocytosed and killed *P. mirabilis* in a 2-h assay in the presence of 10% serum from normal mice. The serum factors supporting phagocytic killing were heat-labile (50°C or 56°C for 30 min) and could be absorbed at 37°C but not 4°C by three different species of Gram-negative bacteria. The tested species of Gram-positive bacterium did not absorb the activity. At the end of the assays, >90% of leucocyte-associated bacteria were associated with neutrophils. Leucocytes from unstimulated peritoneal cavities (<1% neutrophils) did not kill bacteria in this assay, in contrast to leucocyte suspensions containing up to 98% neutrophils. These findings indicated that the phagocytic killing of *P. mirabilis* in this assay was mediated by neutrophils, and that complement fixation by the alternative pathway provided necessary opsonins in normal mouse serum.

Keywords: neutrophils, phagocytic killing, opsonization, complement

In studies of infection and immunity, mice are often chosen as hosts for models of infectious disease, such as intra-abdominal (Shapiro *et al.* 1982; Nulsen *et al.* 1983) and subcutaneous (Joiner *et al.* 1980; Reznikov & McDonald 1983) abscess formation, in the pathogenesis of which neutrophils are key cells. Murine neutrophils are known to possess complement receptors (Lay & Nussenzweig 1968; Lopez *et al.* 1981) and Fc receptors for subclasses of murine IgG (Lopez *et al.* 1981). There are subpopulations of murine neutrophils differentiated on the basis of cell surface Ia antigen expression (Fitzgerald *et al.* 1983). Murine neutrophils also have active halogenating mechanisms (Simmons & Karnovsky 1973), respond to chemotactic stimuli (Snyderman *et al.* 1971), and can phagocytose *in vitro* red blood cells (Mantovani 1975), and organisms such as *Candida albicans* (Morelli & Rosenberg 1971), *Pseudomonas aeruginosa* (Jones & Dyster 1973), *Streptococcus pneumoniae* (Winkelstein *et al.* 1975), and *Salmonella typhimurium* (Baron & Proctor 1982) that have been opsonized in normal serum (NS) from mice. In contrast to studies with human neutrophils (Leist-Welsh & Bjornson 1979), the properties of the opsonins in mouse NS for phagocytic killing by neutrophils have not been fully elucidated. We present evidence that the nature of the opsonin in NS for phagocytic

killing of *Proteus mirabilis* is consistent with the properties of the alternative pathway of complement fixation.

Materials and methods

Mice. Male mice, of the BALB/c strain, aged 4 to 12 weeks, were used. They were housed according to the guidelines of the National Health and Medical Research Council of Australia.

Bacteria. Bacteria other than *Staphylococcus epidermidis* (isolated from the intestinal flora of BALB/c mice) were isolated from intra-abdominal abscesses induced in mice by a complex inoculum of mouse colonic and caecal contents (Nulsen *et al.* 1983). *Proteus mirabilis*, *Escherichia coli* and *S. epidermidis* were identified by the API 20E system (Montalieu-Vercieu, France), and *Bacteroides fragilis* by the Microbact 24A anaerobe system (Disposable Products Ltd, Adelaide, South Australia).

Log-phase *P. mirabilis* for the phagocytic assay was prepared by aerobic growth for 3 to 4 h of a small inoculum of organisms from a plate culture into trypticase soy broth—TSB (BBL, Cockeysville, MD, USA). The viable count was assessed from predetermined standard curves by measuring absorbance at 420 nm in a spectrometer (Spectronic 20, Bausch and Lomb, Rochester, NY, USA). The bacteria were washed in mouse osmolality phosphate-buffered saline—MPBS (Sheridan & Finlay-Jones 1977), pelleted (1500 g, 20 min, 4°C), and suspended in RPMI 1640 medium (Flow Laboratories, McLean, VA, USA) to a concentration of 5×10^6 cfu/ml.

Cultures of bacteria for serum absorption were grown aerobically overnight in TSB, except for *B. fragilis* which was grown overnight in Schaedler's broth (BBL) in an anaerobic chamber.

Cells. Leucocytes, predominantly neutrophils, were harvested from the peritoneal cavities of mice that had been given an intraperitoneal (i.p.) inoculation of 1 ml of brain–heart infusion broth—BHIB (Oxoid, Basingstoke, England) 3 h previously. Mice were killed by cervical dislocation, and the peritoneal cavities washed out with two rinses of 5 ml of MPBS. The cells were pelleted (175 g, 8 min, 4°C) and washed with MPBS before suspension in RPMI 1640 at a concentration of 5×10^7 cells/ml. The RPMI 1640 was buffered with a final concentration of 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid (HEPES; Sigma, St Louis, MO, USA).

Sera. Blood was collected from the retro-orbital venous plexus of normal mice under ether anaesthesia. Pooled blood was allowed to clot on ice for 10 to 30 min. Approximately 1.5 g of Sera Sieve (Hughes and Hughes Ltd, Essex, England) were layered on top, and the blood centrifuged (4000 *g*, 10 min, 4°C). Serum was collected, filtered at 0.22 μm (Millipore, Bedford, MA, USA), and used immediately or snap-frozen in liquid N_2 and stored at −70°C. The resulting serum was termed normal serum (NS). Heat-inactivated serum (HNS) was prepared, unless indicated otherwise, by incubating NS at 56°C for 30 min. Batches of absorbed sera (ANS) were prepared by adding approximately 10^{10} cfu to aliquots of 1 ml NS and incubating the mixtures at 37°C or on ice for 30 min. The bacteria were removed by centrifugation (1500 *g*, 20 min, 4°C) and filtered.

Phagocytic killing assay. The assay mixture consisted of 5×10^6 leucocytes, 5×10^5cfu *P. mirabilis* and 10% serum in a final volume of 1 ml of HEPES-buffered RPMI 1640. The sera tested were NS, HNS, and different batches of ANS, the latter having been absorbed at 37°C or on ice with *P. mirabilis*, *E. coli*, *B. fragilis* or *S. epidermidis*. Control tubes for each serum tested excluded leucocytes. Other controls excluded serum, or both serum and leucocytes. Experiments were carried out aerobically in 15 ml polycarbonate plastic tubes (Disposable Products, South Australia) incubated in a 37°C reci-

procating water bath, with shaking at 110 to 120 oscillations per min through a 5-cm stroke.

Samples of 0.05 ml were removed at 0 h and at various intervals thereafter (usually at 1 and 2 h) for the enumeration of viable bacteria, assessed after disruption of the leucocytes in 5 ml of 0.1% Triton X100 (Ajax Chemicals, Sydney, Australia) in 0.9% saline at room temperature (approximately 22°C). Subsequent serial 10-fold dilutions were made in 0.9% saline, 0.1-ml volumes spread onto CLED agar (Oxoid), and colonies counted after overnight aerobic incubation at 37°C. The concentration of viable *P. mirabilis* in each tube was determined at 0, 1 and 2 h. Two parameters were calculated:

(i) Δ LOG_{10} (cfu/ml) =
LOG_{10} (cfu/ml) at time t
$-LOG_{10}$ (cfu/ml) at time 0

(ii) Leucocyte-associated killing (LAK) =
(Δ LOG_{10} (cfu/ml) in absence of leucocytes)
$-$(Δ LOG_{10} (cfu/ml) in presence of leucocytes)

The first calculated the change in the viable count of *P. mirabilis* from time 0; the second calculated the difference in the viable count associated with the presence of leucocytes under the tested condition (e.g. the presence of NS). A positive value indicated that the viable count was diminished by the presence of leucocytes. The units for both parameters were LOG_{10} (cfu/ml). Additional 0.05-ml samples were removed at 0 h and at various intervals thereafter for the determination of leucocyte morphology and leucocyte-associated bacteria (LAB). The term 'leucocyte-associated' was used in preference to 'phagocytosed', because although the LAB appeared to be intracellular, the possibility that some were on the cell surface could not be excluded. Centrifuged smears (Shandon Cytospin, Cheshire, England; 54 *g*, 5 min) were stained (Jenner–Giemsa stain), and examined by light microscopy. A minimum of 300 cells were classified morphologically and their associated bacteria counted.

Gradient enrichment of neutrophils. A single-step method employing discontinuous density gradients (Penttila *et al.* 1982) was used for the preparation of populations of cells that were usually 98% neutrophils.

Indirect immunofluorescence assay. Approximately 10^5 *P. mirabilis* were placed in each well of 15-well multitest slides (Flow Laboratories, Annandale, N.S.W., Australia), air-dried, heat-fixed and stored at -20°C until used in indirect immunofluorescence assays. In brief, two-fold dilutions of serum were made in PBS and applied to the fixed smears. The slides were incubated at 37°C for 30 min. After washing, the smears were treated (30 min, 37°C) with fluorescein-conjugated goat antiserum to mouse gamma globulin (E.Y. Laboratories, San Mateo, CA, USA). After washing and mounting, the slides were examined by microscopy for fluorescence.

Results

Phagocytic killing required serum

In a series of 11 experiments, the phagocytic killing of *P. mirabilis* by murine neutrophils proceeded in the presence of normal serum, but not in the absence of serum (Fig. 1). The ability of normal serum to support phagocytic killing was abrogated by heating at 56°C for 30 min (Fig. 1), although the presence of leucocytes diminished the growth of *P. mirabilis* in HNS.

Nature of serum factor

The nature of the serum factor supporting phagocytic killing was examined by subjecting NS to various treatments before incorporating it in the assay. In a series of six experiments, heating at 50°C or 56°C for 30 min diminished the ability of NS to support phagocytic killing (Fig. 2) It was also diminished by absorption with *P. mirabilis*, *E. coli*, and *B. fragilis* at 37°C but not at 4°C (Fig. 3). It was not significantly diminished by absorption with *S. epidermidis* at either 4°C or 37°C, when compared with the relevant control, i.e., NS incubated at the

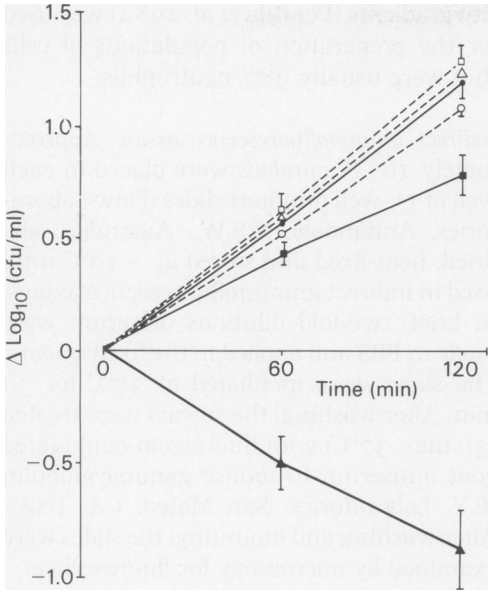

Fig. 1. Changes in the viable counts of *P. mirabilis* in the presence (solid lines and closed symbols) or absence (dashed lines and open symbols) of leucocytes, and in the absence of serum (circles) or in the presence of 10% NS (triangles) or 10% HNS (squares). Each point represents the mean ± s.e.m. of 11 experiments. Neutrophils were 74 ± 5% (mean ± s.e.m.) of the leucocytes in these experiments.

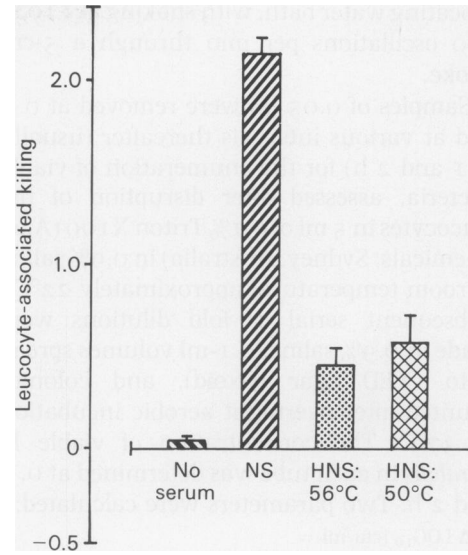

Fig. 2. Leucocyte-associated killing (at 120 min) of *P. mirabilis* in the absence of serum, and in the presence of 10% NS, 10% NS heated at 56°C for 30 min, or 10% NS heated at 50°C for 30 min. The histograms represent the mean ± s.e.m. of six experiments. Neutrophils were 73 ± 6% (mean ± s.e.m.) of the leucocytes in these experiments.

appropriate temperature and time in the absence of bacteria (Fig. 3).

Normal serum antibody titres to P. mirabilis

Titres of antibody to *P. mirabilis* in various NS batches were assayed by indirect immunofluorescence. Sixteen batches of NS had a median titre of 1/2 (mean: 1/2.2; range < 1/2 to 1/8). Aliquots of batches of NS, absorbed with *P. mirabilis* at 4°C, had no detectable antibody to that organism by indirect immunofluorescence.

Phagocytic killing by normal peritoneal cells

The ability of leucocytes (< 1% neutrophils, obtained from unstimulated peritoneal cavities of male BALB/c mice) to phagocytose and kill *P. mirabilis* in this assay system was tested in a series of five experiments in which elicited peritoneal leucocytes (73 ± 4% neutrophils) were concurrently tested with the same batch of NS. Unstimulated peritoneal leucocytes did not kill *P. mirabilis*, in contrast to the neutrophil-enriched population (Fig. 4).

Leucocyte-associated bacteria (LAB).

The bacteria associated with leucocytes at the end of the 2 h assay were determined by examining cytocentrifuge-prepared smears. In a series of five experiments, in excess of 90% of leucocyte-associated bacteria were in/on neutrophils (Table 1).

Gradient-enriched neutrophil suspensions

It was possible, using discontinuous density gradients, to obtain neutrophil suspensions that were 98% pure. These neutrophil-enriched suspensions were not significantly

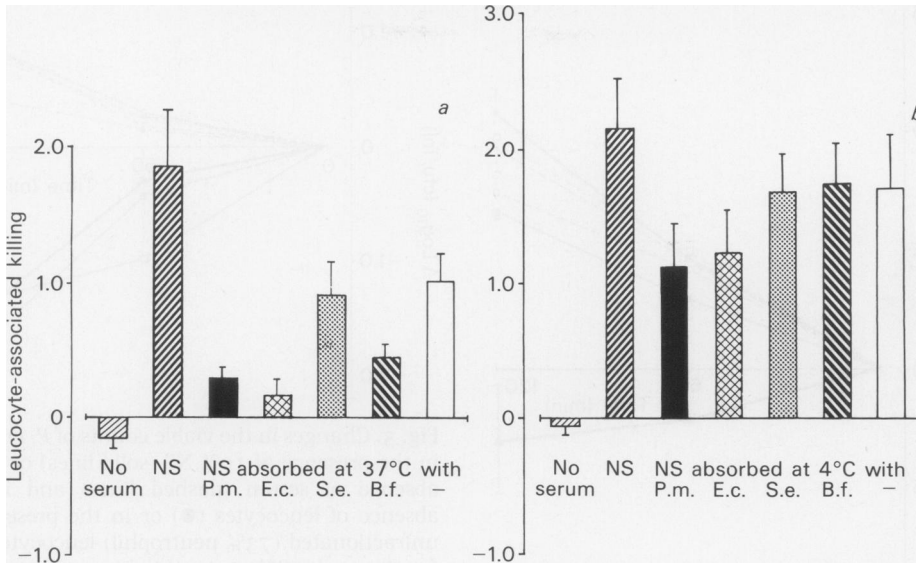

Fig. 3. Leucocyte-associated killing (at 120 min) of *P. mirabilis* in the presence of sera absorbed with various bacteria at 37°C(*a*) or at 4°C(*b*). With respect to the absorptions of sera: P.m., *P. mirabilis*; E.c., *E. coli*; B.f., *B. fragilis*; S.e., *S. epidermidis*; —, no bacteria. Control assays comprised tubes with no serum, or with (unabsorbed) NS. Histograms in (*a*)—absorption at 37°C—represent the mean ± s.e.m. of five experiments. Neutrophils represented 76 ± 4% (mean ± s.e.m.) of the leucocytes present. Histograms in (*b*)—absorption at 4°C—represent the mean ± s.e.m. of four experiments. Neutrophils represented 72 ± 2% (mean ± s.e.m.) of the leucocytes present.

different to the unenriched peritoneal exudate cells in their ability to kill *P. mirabilis*. The results of one experiment are shown (Fig. 5). The experiment was repeated twice with similar results.

Discussion

Murine neutrophils have been used to study the phagocytosis (Jones & Dyster 1973) and phagocytic killing (Bjornson & Michael 1971) of *Pseudomonas aeruginosa*. In the former study, sera from normal and vaccinated mice were used as sources of opsonins, but the nature of the opsonin in normal serum was not examined. In the latter study, the opsonins were human antibodies plus normal mouse serum. Peripheral blood leucocytes (presumably neutrophils) were able to phagocytose *Candida albicans* opsonized in NS better than unopsonized yeast (Morelli & Rosenberg 1971). With the use of acridine orange to differentiate live from dead organisms, the phagocytic killing of *Salmonella typhimurium* was demonstrated with murine neutrophils (Baron & Proctor 1982). The bacteria in this system had been opsonized in 10% NS, but the nature of the opsonins was not probed. A heat-labile, cobra venom factor-inactivated opsonin for neutrophil-mediated killing of *E. coli* has been noted in mice (Gross *et al.* 1978). The phagocytosis of *Streptococcus pneumoniae* by murine neutrophils has been studied *in vitro* and *in vivo* with a morphological assay, and a cobra venom factor-inactivated opsonin in normal serum was described (Winkelstein *et al.* 1975). The results reported in this paper indicate (i), phagocytosis of *P. mirabilis* is enhanced in, but does not have an absolute requirement for, normal serum (Table 1), and, (ii), that normal serum is necessary for

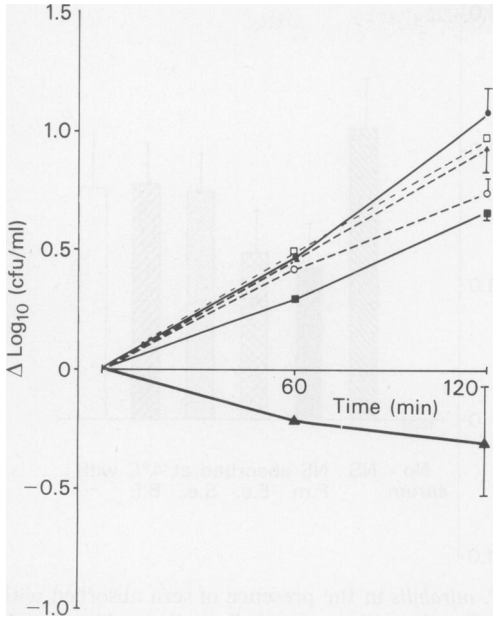

Fig. 4. Changes in the viable counts of *P. mirabilis* in the presence (solid lines and closed symbols) or absence (dashed lines and open symbols) of NS, and in the absence of leucocytes (circles) or in the presence of leucocytes from unstimulated peritoneal cavities (squares) or from peritoneal cavities each given an injection of 1 ml of BHIB 3 h previously (triangles). Each point represents the mean ± s.e.m. of five experiments. Neutrophils were < 1% of the leucocytes in the 'unstimulated' populations, and 73 ± 2% (mean ± s.e.m.) of the leucocytes in the 'stimulated' populations.

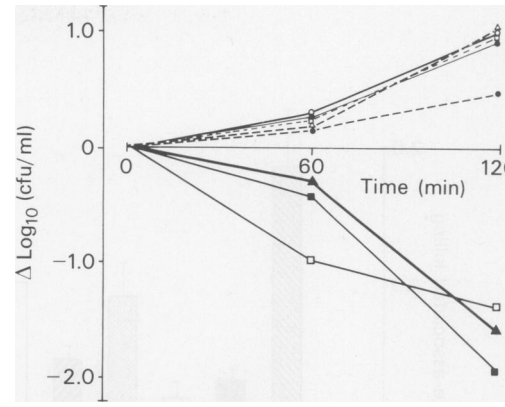

Fig. 5. Changes in the viable counts of *P. mirabilis* in the presence of 10% NS (solid lines) or in the absence of serum (dashed lines) and in the absence of leucocytes (●) or in the presence of unfractionated (73% neutrophil) leucocytes (■), fractionated (98% neutrophil) leucocytes (▲), and in the remixed (86% neutrophil) population (□).

phagocytic killing of the organism by murine neutrophils, and the factor has properties consistent with those of the alternative pathway of complement fixation (Figs 1–3).

The serum component that supported phagocytic killing was labile to heating at 56°C and 50°C (Figs 1 & 2), could be absorbed by various species of Gram-negative bacteria at 37°C but not at 4°C (Fig. 3), but could not be absorbed by the Gram-

Table 1. Leucocyte-associated bacteria

Serum in assay	LAK at 2 h	LAB in neutrophils (%)*	Neutrophils with LAB (%)†
None	−0.22 ± 0.21	93.5 ± 6.5	13.3 ± 6.0
10% NS	1.17 ± 0.21	99.6 ± 1.0	29.2 ± 10.9
10% HNS	0.60 ± 0.28	97.6 ± 3.1	17.8 ± 3.9

Results are mean ± s.d. of five experiments.

LAK, Leucocyte-associated killing; LAB, leucocyte-associated bacteria (determined by light microscopy).

* (No. of LAB in neutrophils)/(no. of LAB in neutrophils and monocytes) × 100.

† (No. of neutrophils with LAB)/(total no. of neutrophils) × 100.

positive bacterium tested (Fig. 3). These properties correlate with those of the alternative pathway of complement fixation. Opsonization of bacteria by the C3 component of complement fixed by either the classical or alternative pathways is markedly inhibited by heating serum at 56°C for 30 min (Stossel 1975; Horwitz 1982). Factor B of the human alternative complement pathway is inactivated by heating at 50°C (Gotze & Muller-Eberhard 1971). Complement fixation pathways in mouse serum are sensitive to heating at 56°C for 30 min. (Rosenberg & Tachibana 1962; Joiner *et al.* 1979; Van Dijk *et al.* 1980), and the murine alternative pathway is sensitive to 50°C (Joiner *et al.* 1979; Van Dijk *et al.* 1980). The lipopolysaccharides of Gram-negative bacteria are able to activate human (Gewurz *et al.* 1968; Gotze & Muller-Eberhard 1971) and mouse (Williams & Wemyss 1961) complement by the alternative pathway.

There were three lines of experimentation suggesting neutrophils were the predominant cell type in phagocytic killing in this assay. Examination of cytocentrifuged smears of the assay mixture after 2 h indicated that >90% of the LAB were in/on neutrophils (Table 1). Peritoneal cells (<1% neutrophils) did not kill in this system (Fig. 4). Elicited peritoneal cells enriched for neutrophils such that the mononuclear component was 2% were able to kill *P. mirabilis* insignificantly differently from the unfractionated, elicited, peritoneal cell population containing 27% monocytes (Fig. 5).

NS supports the phagocytosis and killing of bacteria by mouse macrophages (Jenkin & Benacerraf 1960), in agreement with our results where NS supports phagocytosis and killing by mouse neutrophils.

The phagocytic killing of bacteria by human neutrophils has been studied more extensively. Heterogeneity exists amongst the tested strains of various species of aerobic Gram-negative bacteria: either classical or alternative complement pathways, with or without the participation of antibody, may provide the opsonins in normal human serum (Leist-Welsh & Bjornson 1979). In our studies in mice, there were two pieces of evidence against the significant participation of antibody in opsonization of bacteria by NS. The titre of antibody in NS to *P. mirabilis* was low, and was below levels of detection following absorption at 4°C, yet the ANS was able to support phagocytic killing (Fig. 3*b*). HNS did not support phagocytic killing (Figs 1 & 2), although the growth of *P. mirabilis* in HNS was diminished by the presence of leucocytes (Fig. 1).

The finding that phagocytic killing (Fig. 1), in contrast to phagocytosis (Table 1), of *P. mirabilis*, had an absolute requirement for NS may be explained by the results of Leijh *et al.* (1981) who found using human neutrophils, that extracellular serum was necessary for the optimal intracellular kill of ingested organisms.

Further elucidation of the activity of murine neutrophils against bacteria, and of the nature of the serum opsonins they require, will improve our understanding of the host–pathogen interaction in a commonly used species for models of microbial sepsis.

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